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ORIGINAL MANUSCRIPT

Oral intake of ranitidine increases urinary excretion of *N*-nitrosodimethylamine

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Abstract

The H_2-receptor antagonist, ranitidine, is among the most widely used pharmaceuticals to treat gastroesophageal reflux disease and peptic ulcers. While previous studies have demonstrated that amines can form *N*-nitrosamines when exposed to nitrite at stomach-relevant pH, *N*-nitrosamine formation from ranitidine, an amine-based pharmaceutical, has not been demonstrated under these conditions. In this work, we confirmed the production of *N*-nitrosodimethylamine (NDMA), a potent carcinogen, by nitrosation of ranitidine under stomach-relevant pH conditions *in vitro*. We also evaluated the urinary NDMA excretion attributable to ingestion of clinically used ranitidine doses. Urine samples collected from five female and five male, healthy adult volunteers over 24-h periods before and after consumption of 150 mg ranitidine were analyzed for residual ranitidine, ranitidine metabolites, NDMA, total *N*-nitrosamines and dimethylamine. Following ranitidine intake, the urinary NDMA excreted over 24 h increased 400-folds from 3.0 to 47 000 ng, while total *N*-nitrosamines increased 5-folds. NDMA excretion rates after ranitidine intake equaled or exceeded those observed previously in patients with schistosomiasis, a disease wherein *N*-nitrosamines are implicated as the etiological agents for bladder cancer. Due to metabolism within the body, urinary NDMA measurements represent a lower-bound estimate of systemic NDMA exposure. Our results suggest a need to evaluate the risks attributable to NDMA associated with chronic consumption of ranitidine, and to identify alternative treatments that minimize exposure to *N*-nitrosamines.

Introduction

Ranitidine, sold under the trade name Zantac®, is a histamine H_2-receptor antagonist commonly used to treat gastroesophageal reflux disease and gastric and duodenal ulcers (1,2). Zantac® was the top over-the-counter H_2-receptor brand in the USA in 2013 (3), and is consistently ranked among the top 20 sold-list of prescribed drugs in several European countries and Australia (4). Previous research (5–9) has demonstrated that certain pharmaceuticals containing amine moieties can form *N*-nitrosamines via reactions with nitrite at stomach-relevant pH *in vitro* [i.e. endogenous nitrosation; Equations 1 and 2 for formation of *N*-nitrosodimethylamine (NDMA) from dimethylamine (DMA)]. *N*-Nitrosamines are considered likely human carcinogens (10–13), and have been implicated in the induction of stomach, esophagus and nasopharynx cancers (14). Additionally, considerable evidence indicates *N*-nitrosamines as the etiological agents for bladder cancer associated with schistosomiasis (15).

$$2HNO_2 \leftrightarrow N_2O_3 + H_2O \quad (1)$$

$$N_2O_3 + (CH_3)_2NH \rightarrow (CH_3)_2N{-}N{=}O + HNO_2 \quad (2)$$

To the best of our knowledge, no previous study has demonstrated conversion of ranitidine to NDMA at stomach-relevant pH. Only scant, and inconclusive data exists for the impact of ranitidine

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Abbreviations

DMA	dimethylamine
NDMA	*N*-nitrosodimethylamine
PPI	proton pump inhibitor
TONO	total *N*-nitrosamines

intake on *N*-nitrosamine levels in human gastric juice (16–18). Thomas *et al.* (17) found no significant increase in the median daytime intragastric *N*-nitrosamine levels in peptic ulcer patients receiving ranitidine treatment compared to those who undertook truncal vagotomy. Garcia del Risco *et al.* (18) also reported no statistically significant rise in the mean intragastric *N*-nitrosamine concentrations in healthy adults taking ranitidine. However, Matsuda *et al.* (16) found that NDMA and *N*-nitrosodiethylamine concentrations in gastric juice doubled between gastric ulcer patients taking ranitidine compared to those who were not given ranitidine. Because *N*-nitrosamines also can form by other mechanisms within the body (19,20), it is difficult to estimate systemic exposure from intragastric concentrations. Measurements of *N*-nitrosamine concentrations in urine would provide a better estimate of systemic exposure, albeit conservative given metabolism of *N*-nitrosamines within the body (21). Previous research has not measured *N*-nitrosamine excretion in urine following ranitidine intake.

To enable better estimates of systemic exposure to *N*-nitrosamines from ranitidine intake, the goal of this study was to evaluate the impact of ranitidine intake on urinary excretion of *N*-nitrosamines, with a particular emphasis on NDMA, the *N*-nitrosamine most structurally similar to the nitrosatable dimethylamino functional group in ranitidine. We demonstrated the formation of NDMA and other ranitidine transformation products under acid-nitrite conditions in the laboratory, established the 24-h profiles as well as the means and ranges of specific and total *N*-nitrosamine masses and concentrations excreted in the urine of 10 healthy adult volunteers (five females and five males) just before and after consumption of one Zantac 150® tablet. We validated the associations between ranitidine intake and urinary excretion of *N*-nitrosamines, ranitidine and dimethylamine. Human exposure to *N*-nitrosamines also includes intake of preformed *N*-nitrosamines from dietary (e.g. foods, drinking water), environmental (e.g. tobacco products) and occupational sources as well as endogenous formation of *N*-nitrosamines from sources other than ranitidine (19), providing a baseline *N*-nitrosamine exposure beyond pharmaceutical consumption. By comparing urinary *N*-nitrosamine excretion before and after ranitidine intake, we demonstrated that ranitidine consumption increases NDMA exposure 400-fold and total *N*-nitrosamine exposure 5-fold above this baseline exposure. Given the widespread use of ranitidine, our results suggest the need to better characterize cancer risks associated with chronic ranitidine consumption, and to identify alternative treatments that reduce *N*-nitrosamine exposure.

Materials and methods

The supplementary material provides chemical sources and purity.

In vitro ranitidine nitrosation assays

In vitro ranitidine nitrosation was first performed following the same protocol as Maura *et al.* (22) for initial screening of transformation products. Five mmol of sodium nitrite ($NaNO_2$) dissolved in ultrapure water (5 ml of 1M) was added dropwise with constant stirring to 2N hydrochloric acid (HCl; 15 ml, pH < 1) containing 2.5 mmol of ranitidine (~0.877 g ranitidine hydrochloride) cooled to 4°C in an ice bath. The reaction mixture was incubated in a thermostated water bath at 37°C in the dark for 24 h, adjusted to pH 10 [8 ml of 20 wt% potassium carbonate (K_2CO_3) solution], and extracted with anhydrous diethyl ether (15 ml × 3). The combined ether extract was repeatedly washed with saturated sodium chloride solution (15 ml × 3), dried over anhydrous K_2CO_3 and evaporated to dryness under N_2 to yield a yellow oil, as reported in Maura *et al.* (22) The remaining alkaline solution was extracted with chloroform (15 ml × 3) and retained. The combined chloroform extract was dried over K_2CO_3 and evaporated to dryness to yield a light orange solid. The alkaline solution retained from the chloroform extraction was further extracted with dichloromethane (DCM; 15 ml × 3) and again retained. The combined DCM extract was dried over K_2CO_3 and evaporated to dryness to yield an orange solid. Lastly, the alkaline solution retained from the DCM extraction was neutralized with 1N HCl and evaporated to dryness under high vacuum to yield a dark orange solid (23). Individual extracts (~1 mg) were redissolved in 10 ml of 0.1% (v/v) formic acid and a 5-µl aliquot was analyzed using liquid chromatography quadrupole-time-of-flight high-resolution mass spectrometry (i.e. LC-QToF-HRMS). Method details for LC-QToF-HRMS analysis are provided in the Supplementary material, available at *Carcinogenesis* Online.

Additional ranitidine nitrosation experiments were performed to evaluate the effect of pH and nitrite-to-ranitidine molar ratio, respectively, on product formation under simulated gastric conditions similar to those described by Mirvish (2[illegible]nd Lijinsky (16). Fifty µmol of ranitidine (0.1 ml of 500 mM ranitidine hydrochloride) was mixed with an aliquot of ultrapure water adjusted to a predetermined pH with 0.1N HCl. Different volumes of $NaNO_2$ solution (0–1[illegible]0 µl of 500 mM) were added dropwise with constant stirring to the acidic ranitidine solutions (pH 3.01) to achieve a range of nitrite-to-ranitidine molar ratios (i.e. 0, 0.1, 0.2, 0.5, 1, 2, 5 and 10 mmol/mmol) and a constant final reaction volume of 10 ml (i.e. 5 mM ranitidine). Similarly, different volumes of 0.1N HCl (0–1000 µl) were added to the acidic ranitidine solutions (with a fixed $NaNO_2$-to-ranitidine molar ratio of 5) to achieve a range of pH (i.e. 2.03, 2.52. 3.01, 3.52, 4.06 and 5.25). The reaction mixtures were incubated in a thermostated water bath at 37°C in the dark for 24 h. A 5-µl aliquot of samples was analyzed using liquid chromatography triple-quadrupole mass spectrometry (i.e. LC-QqQ-MS) to track the disappearance and growth of chromatographic peaks, as detailed below (Supplementary Table 3, available at *Carcinogenesis* Online). A second 100-µl aliquot of samples was derivatized with 2,3,4,5,6-pentafluorobenzoyl chloride and analyzed for dimethylamine (DMA) using the same LC-QqQ-MS system, as detailed below. A third 9-ml aliquot of samples was extracted by liquid–liquid extraction with DCM and analyzed for NDMA using gas chromatography ion trap mass spectrometry (i.e. GC-IT-MS) as described previously (25).

Urine collection and sample preparation

Urine samples were collected from 10 adults (five men and five women; ages 20–49 years). None of the volunteers smoked, consumed alcohol or had known gastrointestinal symptoms, renal diseases or urinary infections. Urine collection and handling protocols were approved by the Stanford University Institutional Review Board (Protocol Number 32245) and informed written consent was obtained from each volunteer. Each volunteer collected a series of urine samples using Commode specimen collection containers over a 48-h period before and after oral administration (i.e. at the 24th hour) of one Zantac 150® tablet (150 mg active ingredient; Boehringer Ingelheim Pharmaceuticals, Inc., Ridgefield, CT). Each voiding was collected in a separate collection container, and thus the specific collection times varied among the volunteers. Immediately after collection, urine samples were preserved with sodium hydroxide pellets to halt nitrosation and bacterial growth (26) and stored at –20°C in the dark until analysis. Selected urine samples were spiked with morpholine (100 µg/l) to monitor any artefactual formation of *N*-nitrosomorpholine (NMOR) during sample storage and/or processing (27).

N-Nitrosamine analysis in urine

For the analysis of specific *N*-nitrosamines, an aliquot of raw urine sample (20 ml) was spiked with *N*-nitrosodimethylamine-d_6 (NDMA-d_6; 5 µl of 2 mg/l) and *N*-nitrosomorpholine-d_8 (NMOR-d_8; 5 µl of 2 mg/l) as internal standards and extracted with DCM (20 ml) by vortexing for 2 min. Following extraction, the sample was centrifuged in a Thermo Sorvall Legend XT centrifuge (8000*g* for 5 min) and the upper aqueous phase was discarded. The lower DCM phase was centrifuged (10 000*g* for 5 min) to facilitate removal of residual aqueous droplets, dried over anhydrous

K_2CO_3 and further concentrated under a gentle stream of nitrogen (N_2) to a final volume of ~500 µl. A 5-µl aliquot of the DCM extract was analyzed using GC-IT-MS as described previously (25). Nine specific *N*-nitrosamines were targeted, including NDMA, *N*-nitrosomethylethylamine (NMEA), *N*-nitrosodiethylamine, *N*-nitrosodi-*n*-propylamine (NDPA), *N*-nitrosodi-*n*-butylamine (NDBA), *N*-nitrosopyrrolidine (NPYR), *N*-nitrosopiperidine (NPIP), NMOR and *N*-nitrosodiphenylamine. Specific *N*-nitrosamine concentrations in ultrapure water blank samples were below the method detection limits (Supplementary Table 1, available at *Carcinogenesis* Online).

For the analysis of total *N*-nitrosamines (TONO), an aliquot of raw urine sample (1 ml) was treated with sulfamic acid (~100 mg) overnight to pH <2 and amended with mercuric chloride (10 µl of 20 g/l) and sulfanilamide (10 µl of 50 g/l in 1N HCl) to eliminate interferences from *S*-nitrosothiols and nitrite (28). The treated sample was centrifuged in a Beckman Microfuge 18 centrifuge (14 000*g* for 30 min) and a 100-µl aliquot was injected into a heated reaction vessel containing a freshly prepared acidic iodine/iodide solution. The nitric oxide (NO) released from the *N*-nitrosamines was measured using an EcoPhysics CLD 88Yp chemiluminescence detector as described previously (25,29,30). The detector response was calibrated against NDMA, and the raw TONO values were further corrected by subtracting off any detector response that might be attributed to NO liberation from the C-terminal nitro groups on ranitidine and its metabolites (17). Hereafter, TONO concentrations are expressed as NDMA equivalents based in reference to the NDMA standard curve. TONO concentrations in ultrapure water blank samples were below the method detection limit (Supplementary Table 2, available at *Carcinogenesis* Online).

Ranitidine and metabolite analysis in urine

For the analysis of ranitidine and its known metabolites (i.e. ranitidine N-oxide, ranitidine S-oxide and desmethyl ranitidine), an aliquot of diluted urine sample (10 µl in 990 µl of 5 mM ammonium acetate) was spiked with ranitidine-d_6 (10 µl of 10 mg/l) as the internal standard. The spiked sample was centrifuged in a Beckman Microfuge 18 centrifuge (14 000 *g* for 30 min) and a 10-µl aliquot was analyzed using LC-QqQ-MS. Method details for LC-QqQ-MS analysis are provided in the Supplementary Table 3, available at *Carcinogenesis* Online.

Dimethylamine analysis in urine

For the analysis of DMA, an aliquot of diluted urine sample (100 µl in 900 µl of 5 mM NH_4OAc) was spiked with dimethylamine-d_6 (DMA-d_6; 10 µl of 10 mg/l) as the internal standard. The spiked sample was amended with K_2CO_3 (50 µl of 20 mM) and derivatized with 2,3,4,5,6-pentafluorobenzoyl chloride (10 µl of 10 wt% in acetonitrile) by vortexing for 1 min as described by Tsikas *et al.* (31). Following derivatization, the sample was centrifuged (14 000 *g* for 3 min) and a 10-µl aliquot was analyzed using LC-QqQ-MS. Method details for LC-QqQ-MS analysis are provided in the Supplementary Table 4, available at *Carcinogenesis* Online.

Statistical analysis

No assumptions were made about data distribution. Statistical significance was tested by the nonparametric *Wilcoxon* matched-pairs signed rank test, with a *P* value of <0.05 considered statistically significant. *Spearman* correlation coefficients and *P* values were computed for correlation analysis. Statistical analysis was performed using *R* 3.2.1 (32).

Results

Ranitidine produces NDMA in the presence of acid and nitrite *in vitro*

While two previous studies suggested the formation of a *N*-nitroso-nitrolic acid product (P372 in Table 1) from ranitidine under acid-nitrite conditions (22,33), no detailed product characterization was conducted to validate this suggestion other than an equivocal colorimetric assay. Furthermore, previous research has not demonstrated NDMA formation from ranitidine under these conditions. Because product characterization is critical to understanding the fate of pharmaceuticals, particularly their potential to generate carcinogens, high-resolution mass spectrometry was used to identify products formed from reactions of ranitidine and nitrite at stomach-relevant pH *in vitro*. Table 1 summarizes products identified using the same conditions as Maura *et al.*(22) (i.e. pH <1, nitrite-to-ranitidine molar ratio of 2:1 for 45 min at room temperature). No *N*-nitroso-nitrolic acid product was found. Instead, 3-(methylamino)-5,6-dihydro-2*H*-1,4-thiazin-2-one *O*-((5-((dimethylamino)methyl)furan-2-yl)methyl) oxime (**P296**) was identified as the dominant product in the diethyl ether extract, accounting for >95% of the total chromatographic peak area. Further extractions with chloroform and dichloromethane revealed three other major products, (5-((dimethylamino)methyl)furan-2-yl)methanol (**P155**), 3-(methylamino)-5,6-dihydro-2*H*-1,4-thiazin-2-one oxime (**P159**), and *N*′-(2-(((5-((dimethylamino)methyl)furan-2-yl)methyl)thio)ethyl)-2-(hydroxyimino)-*N*-methyl-2-nitroacetimidamide, the nitrolic acid derivative of ranitidine (**P343**). P155, P159, and P296 were previously identified by Haywood *et al.* (23) as products formed during the hydrolytic degradation of ranitidine under strongly acidic conditions. Traces (yields <0.1%) of the N-oxide, S-oxide, and desmethyl metabolites of ranitidine were also identified.

Figure 1 presents product formation monitored over a range of pH (i.e. 2.03–5.25) or $NaNO_2$-to-ranitidine molar ratios (i.e. 0–10 mol/mmol) following simulated gastric conditions described by Mirvish (24) and Lijinsky (6) (e.g. reaction for 24 h at 37°C). Due to the lack of authentic standards, products P155, P159, P296 and P343 were reported based on chromatographic peak area ratios relative to ranitidine. At a 5:1 $NaNO_2$-to-ranitidine molar ratio, ranitidine decomposition and P155, P159 and P343 production declined with increasing pH, particularly above pH 3.5. However, P296 formation was maximized at pH 2.52. The concentration of DMA increased from ~39 µM at pH 2.03 to a maximum concentration of ~234 µM at pH 5.25, while NDMA decreased from ~57 µM at pH 2.03 to <0.01 µM at pH 5.25. At pH 3.01, ranitidine decomposition increased with increasing $NaNO_2$-to-ranitidine molar ratios, reaching a maximum of ~30% at molar ratios >1. Note that <0.5% of ranitidine decomposed in the absence of nitrite. With increasing nitrite, DMA yields declined, while NDMA increased, reaching a maximum of ~32 µM. P296 formation increased with nitrite, while P155, P159 and P343 formation were maximized at intermediate molar ratios. Note that P155 and P296, both significant products of ranitidine, retain the dimethylamino functional group associated with NDMA formation. The concentration profiles of DMA suggest that it likely formed as a byproduct of ranitidine nitrosation. Previous research has demonstrated that nitrosation of tertiary amines, such as the dimethylamino-containing moiety in ranitidine (where R refers to the rest of the ranitidine molecule), forms an unstable tertiary *N*-nitrosamine (34,35). This unstable intermediate can undergo dealkylation to release DMA, which in turn can be nitrosated to form NDMA, a stable secondary *N*-nitrosamine:

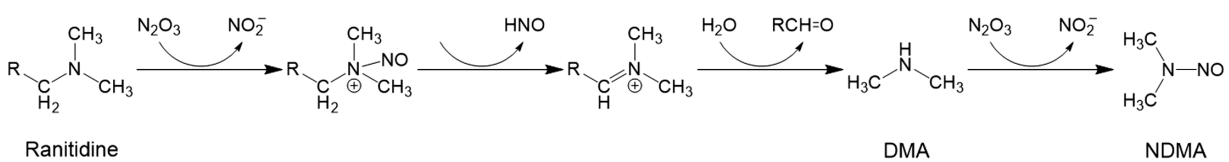
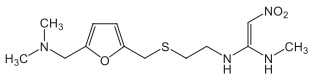
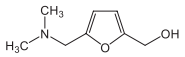
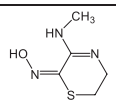
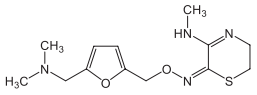
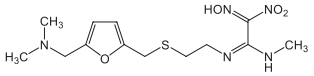
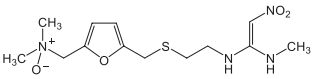
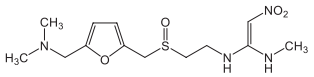
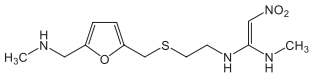
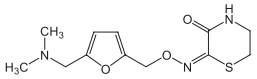
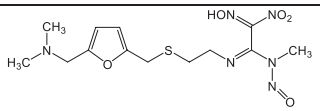


Table 1. High-resolution accurate mass measurements of identified ranitidine nitrosation products

Compound	Formula	Experimental Mass	Theoretical Mass	Accuracy (ppm)	Matching Score	Structure
Ranitidine	$C_{13}H_{22}N_4O_3S$	314.14151	314.14126	0.81	99.50	
P155	$C_8H_{13}NO_2$	155.09466	155.09463	0.18	99.71	
P159	$C_5H_9N_3OS$	159.04662	159.04663	-0.08	99.91	
P296	$C_{13}H_{20}N_4O_2S$	296.13070	296.13092	0.75	95.50	
P343	$C_{13}H_{21}N_5O_4S$	343.13138	343.13142	-0.13	95.08	
P330-A (Ranitidine N-Oxide)	$C_{13}H_{22}N_4O_4S$	330.13540	330.13619	-0.78	94.39	
P330-B (Ranitidine S-Oxide)	$C_{13}H_{22}N_4O_4S$	330.13576	330.13618	-0.42	91.54	
P300 (Desmethyl Ranitidine)	$C_{12}H_{20}N_4O_3S$	300.12640	300.12561	2.63	96.66	
P283[a]	$C_{12}H_{17}N_3O_3S$	283.09714	283.09906	0.28	99.87	
P372[b] (Not found)	$C_{13}H_{20}N_4O_5S$	-	372.12159	-	-	

[a] P283 (2-(((5-((dimethylamino)methyl)furan-2-yl)methoxy)imino)thiomorpholin-3-one), which has not been reported as a ranitidine degradation product, likely formed via hydrolytic cleavage of the methylamine group on P296 by hydroxyl ion during alkaline extraction rather than under acidic nitrite conditions. [b] *N*-Nitroso-nitrolic acid product proposed by Brittain *et al.*(33) and Maura *et al.*(22), which was not found in the current study.

Indeed, the fact that the concentration of NDMA co-varied inversely with that of DMA indicates that the DMA that formed was nitrosated to form NDMA, in accordance with previous work indicating quantitative conversion under similar conditions (36). However, at a $NaNO_2$-to-ranitidine molar ratio of 5:1 at pH 3.01, the yield of NDMA from ranitidine was ~2.5% on a molar basis.

Ranitidine increases urinary excretion rates of NDMA, total *N*-nitrosamines, and DMA

Figure 2 illustrates the urinary excretion profiles of *N*-nitrosamines, dimethylamine (DMA), ranitidine and its metabolites for one male and one female volunteer (the widths of the bars in Figure 2 represent the times between collections of individual urine samples). Note that urinary creatinine levels in both female and male volunteers were not significantly different before and after ranitidine intake (Supplementary Table 5, available at *Carcinogenesis* Online). In both cases, the excretion rates of NDMA, total *N*-nitrosamines (TONO), DMA, ranitidine and its metabolites significantly increased and peaked during the first 3 h following ranitidine intake, but returned to basal levels within the second 24-h monitoring period. Products P155, P159, P296 and P343, detected during *in vitro* nitrosation experiments (Table 1), were not detected. The excretion rate of TONO was consistently higher than that of NDMA. In addition to NDMA, two other *N*-nitrosamines, NPYR and NPIP, were detected in urine, but their excretion rates did not change significantly following ranitidine intake. The excretion rate of ranitidine N-oxide was the highest among the three metabolites, followed by ranitidine S-oxide and desmethyl ranitidine, respectively, which agreed with their relative abundance in urine as reported in previous pharmacokinetic studies (37,38). However, the excretion rates of all three metabolites were

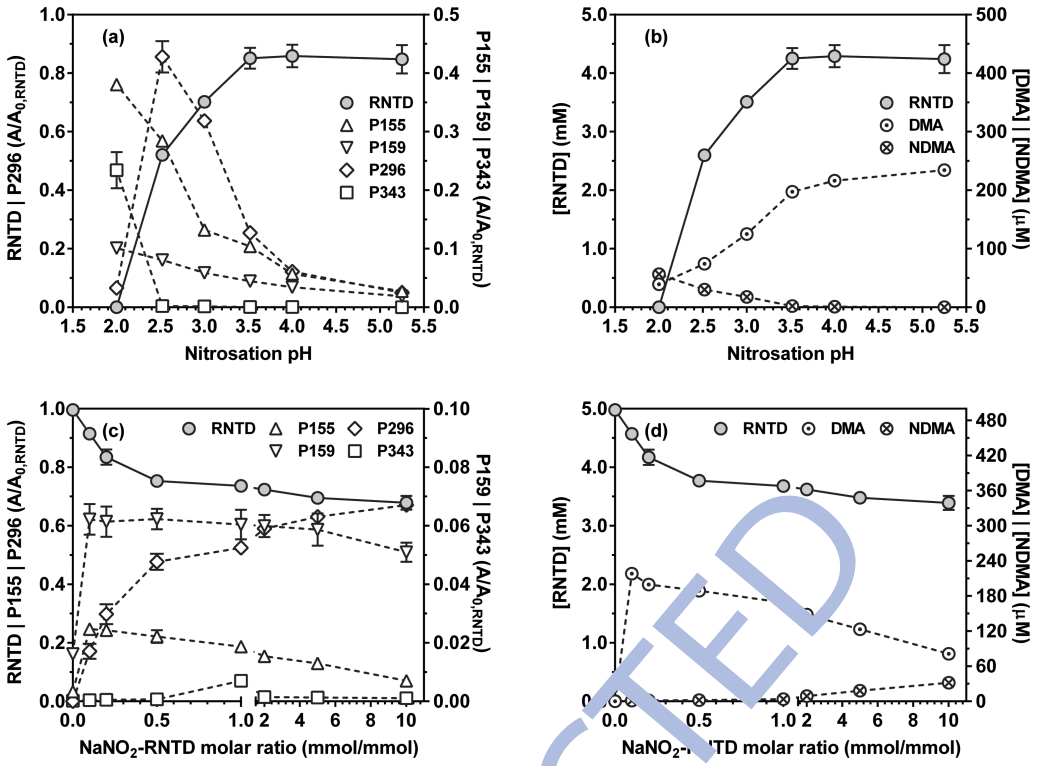


Figure 1. Formation profiles of DMA, NDMA and other products from in vitro nitrosation of 5 mM ranitidine (RNTD) as a function of pH or nitrite-to-ranitidine molar ratios. Error bars represent the range of duplicate analyses; where absent bars fall within symbols.

minor compared to the excretion rates of DMA. DMA is one of the most frequently detected nitrosatable secondary amines in human urine and a major precursor to endogenously formed NDMA (39). Similar excretion patterns were observed for the other eight volunteers (Supplementary Figures 1 and 2, available at *Carcinogenesis Online*) with different age, body weights, and dietary habits.

Figure 3 shows the means and ranges of the 24-h total excreted masses and 24-h mean urinary concentrations of *N*-nitrosamines for all volunteers before and after ranitidine intake (see Supplementary Table 1, available at *Carcinogenesis* Online, for grouped female and male data). No significant differences were observed between females and males (Supplementary Figure 3, available at *Carcinogenesis* Online). The 24-h total excreted masses of *N*-nitrosamines before ranitidine intake concurred with previous research. The 24-h excreted mass of NDMA ranged from 37 to 230 ng with a mean(±SD) of 110 ± 63 ng, comparable to levels reported by Garland *et al.* (38 ± 25 ng) (27), van Maanen *et al.* (11 ± 43 and 89 ± 22 ng) (40,41), and Vermeer *et al.* (290 ± 220 and 380 ± 190 ng) (42,43) in healthy adults. The 24-h excreted masses of NPYR and NPIP were 77 ± 67 and 53 ± 34 ng, respectively, in agreement with the ranges reported by van Maanen *et al.* (91 ± 78 ng for NPYR and 38 ± 78 ng for NPIP) (41) and Levallois *et al.* (67 ± 90 ng for NPIP) (44) in healthy adults. The 24-h excreted mass of TONO ranged from 16 900 to 28 900 ng as NDMA, with a mean of 24 400 ± 4200 ng as NDMA (i.e. 0.33 ± 0.06 μmol), similar to the daily urinary excretion of 1.30 ± 1.05 μmol in healthy adults as estimated by Tricker (19). The 24-h excreted masses of NDMA, NPYR and NPIP only accounted for approximately 0.5, 0.3 and 0.2%, respectively, of the 24-h excreted mass of TONO, suggesting that the majority of TONO consisted of other *N*-nitrosamine species not targeted in this study (e.g. *N*-nitrosoproline and *N*-nitrososarcosine (19)).

24-h excreted mass of DMA prior to ranitidine intake was 9.7 ± 2.8 mg, similar to the mean and range reported by Tricker *et al.* (mean 15.9 mg and range 2.95–72 mg) (39) and by Bouatra *et al.* (mean 20.5 mg and range 13.5–39.3 mg) (45).

After ranitidine intake, the 24-h excreted mass of ranitidine accounted for 33 ± 12% of the 150 mg ranitidine ingested. The 24-h excreted mass of DMA increased over two folds to 24.9 ± 5.2 mg. Dimethylamine was a significant product of ranitidine, accounting for 72 ± 8% of ranitidine degraded on a molar basis. In contrast, the masses of excreted metabolites ranitidine N-oxide, ranitidine S-oxide and desmethyl ranitidine accounted for only 5.7 ± 1.6, 2.4 ± 0.7 and 1.3 ± 0.6% of degraded ranitidine. The 24-h excreted masses of NDMA and TONO increased significantly ($P < 0.05$). NDMA increased by ~400 folds to 47 600 ± 20 700 ng, with the highest mass reaching 74 100 ng in one female. Although a potent carcinogen, NDMA was a minor product, accounting for 0.21 ± 0.10% of degraded ranitidine. The 24-h excreted TONO mass increased over 5-fold to 139 000 ± 26 000 ng as NDMA. The 24-h excreted mass of NDMA amounted to 32 ± 12% of the 24-h excreted TONO mass. Taking the 24-h excreted NDMA mass prior to ranitidine intake as a basal excretion amount, the increase in excreted NDMA mass following ranitidine intake accounted for ~40 ± 13% of the increase in TONO. The 24-h excreted masses of NPYR (80 ± 64 ng) and NPIP (57 ± 38 ng) were not statistically different from those measured before ranitidine intake ($P > 0.05$) and explained only ~0.1% of the 24-h excreted TONO mass.

The relationships between the 24-h urinary data of NDMA, TONO, DMA and ranitidine were further examined by *Spearman* rank correlation analysis (Figure 4). Before ranitidine intake, no significant correlations could be identified between the 24-h total excreted masses or 24-h mean urinary concentrations of NDMA and TONO or DMA ($P > 0.05$; Supplementary Figure 4, available

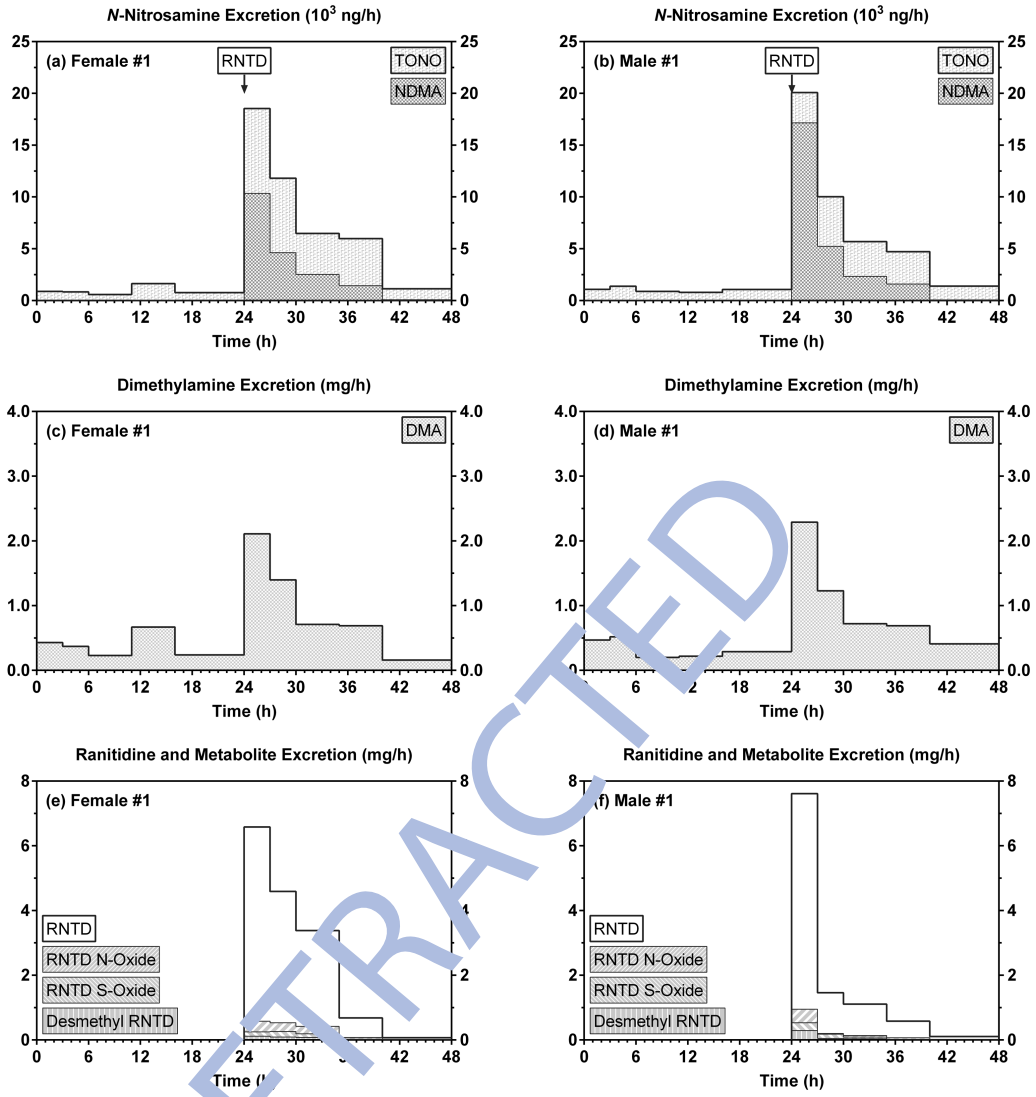


Figure 2. Example urinary excretion ... (i.e. ... female and one male volunteers) of *N*-nitrosodimethylamine (NDMA), total *N*-nitrosamines (TONO), dimethylamine (DMA), ranitidine (RNTD) and its ... metabolites over t... 48-h monitoring period. The excretion rate of *N*-nitrosamines is expressed as '10^3 ng/h'. The excretion profiles of NPYR and NPIP were not shown du... the ... excretion rates. Neither ranitidine nor its metabolites were detected in urine samples collected prior to ranitidine intake.

at *Carcinogenesis* Online). After rani...dine intake, the correlations between the 24-h excreted masses or urinary concentrations of NDMA and TONO were strong with *Spearman* $r > 0.8$ and $P < 0.05$ (Figure 4a and b). The strong positive correlations support the concurrent increases in NDMA and TONO excretion rates (Figure 2) and the finding that NDMA constituted a major fraction of TONO excreted in urine following ranitidine intake. The 24-h mean urinary concentrations of NDMA, TONO and DMA all correlated with that of ranitidine, with *Spearman* $r > 0.7$ and $P < 0.05$ (Figure 4c–e). Furthermore, the 24-h mean urinary concentration of NDMA directly correlated with that of DMA (*Spearman* $r = 0.93$ and $P = 0.0003$; Figure 4f). These correlations suggest that ranitidine either served as a direct precursor for the endogenous formation of NDMA or produced NDMA precursors *in vivo* (e.g. via nitrosative dealkylation to form DMA (34,35)), which were subsequently converted to NDMA. Note that when ranitidine was nitrosated *in vitro*, NDMA formed at the expense of DMA when the nitrosation conditions (e.g. pH and nitrite concentrations) were varied (Figure 1). The correlation between ranitidine, DMA and NDMA urinary concentrations among samples collected from various volunteers does not contradict the *in vitro* results. Instead, these results suggest that in the presence of more ranitidine, concentrations of both DMA and NDMA increase as products of nitrosation.

Discussion

Considerable evidence implicates *N*-nitrosamines as the etiological agents for bladder cancer associated with schistosomiasis (14). We compared our results against urinary NDMA concentrations measured in schistosomiasis patients as a rough estimate for potential risks. Our results indicate that the urinary excretion of NDMA significantly increased following ranitidine intake to levels similar to or even higher than those measured in patients diagnosed with schistosomiasis or bladder cancer. For example, the 24-h excreted mass of NDMA after ranitidine intake (47 600 ± 20 700 ng) was comparable to those reported by Mostafa *et al.* in patients infected with *Schistosoma haematobium* (19 200 ± 21 000 ng) (46). The range was an order of magnitude higher than levels reported by Tricker *et al.* in patients infected with *Schistosoma mansoni* and mixed-type

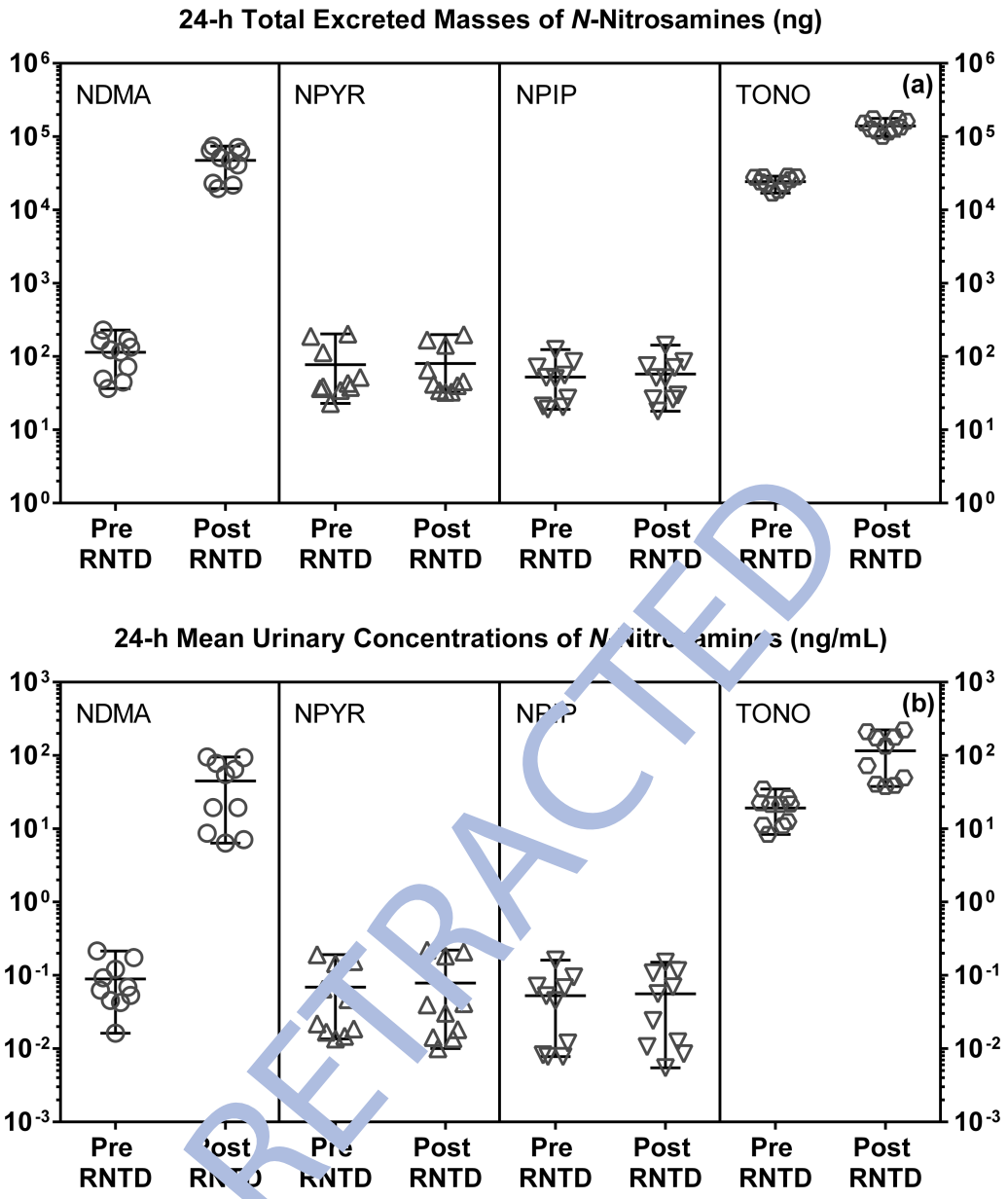


Figure 3. Box-and-Whisker plots of the 24-h total excreted masses and 24-h mean urinary concentrations of N-nitrosamines before (i.e. pre RNTD) and after (i.e. post RNTD) ranitidine intake (n = 10). Note logarithmic scale on the y-axis.

schistosomiasis with or without bladder cancer (1300±1600 and 2700±6100 ng, respectively) (47) and by Abdel Mohsen *et al.* in patients infected with *Schistosoma haematobium* and diagnosed with bladder cancer (6000±2400 and 3200±2000 ng, respectively) (48). Similarly, the 24-h excreted TONO mass after ranitidine intake (139 000±26 000 ng as NDMA) was comparable to the total *N*-nitrosamines reported by Tricker *et al.* in schistosomiasis patients with or without bladder cancer (44 900±7300 ng and 62 900±21 900 ng, respectively) (47). For the purposes of a rough, initial exposure comparison, a typical oral dosage of ranitidine for the treatment of duodenal or gastric ulcer or gastroesophageal reflux is 150 mg twice daily. If the urinary NDMA mass excretion scales with ranitidine dose, 95 200 ng NDMA/day would be excreted in urine. With 3200 ng NDMA/day as the median excretion rate among the schistosomiasis studies, the total NDMA mass excreted from a patient during a 2-month treatment with ranitidine would be comparable to that excreted over 5 years from a schistosomiasis patient (a timescale for untreated *Schistosoma* worms to persist in the body) (49).

In addition to bladder cancer-associated risk, our urinary measurements have broader implications for systemic cancer risk. A previous study had measured a maximum NDMA concentration of 7.9 ng/ml in human gastric juice after ranitidine intake, only a 6.6-fold increase relative to levels measured without ranitidine (i.e. 1.2 ng/ml) (16). In our study, the maximum urinary NDMA concentration was 95.6 ng/ml, 12-fold higher than the maximum gastric NDMA concentration measured in the previous study, and 430-fold higher than the maximum NDMA concentration we measured before ranitidine intake in urine (Supplementary Table 5, available at *Carcinogenesis* Online). The greater increase in concentration measured in urine than measured previously in gastric fluids suggests additional NDMA formation from ranitidine by other pathways within the body after uptake from the stomach

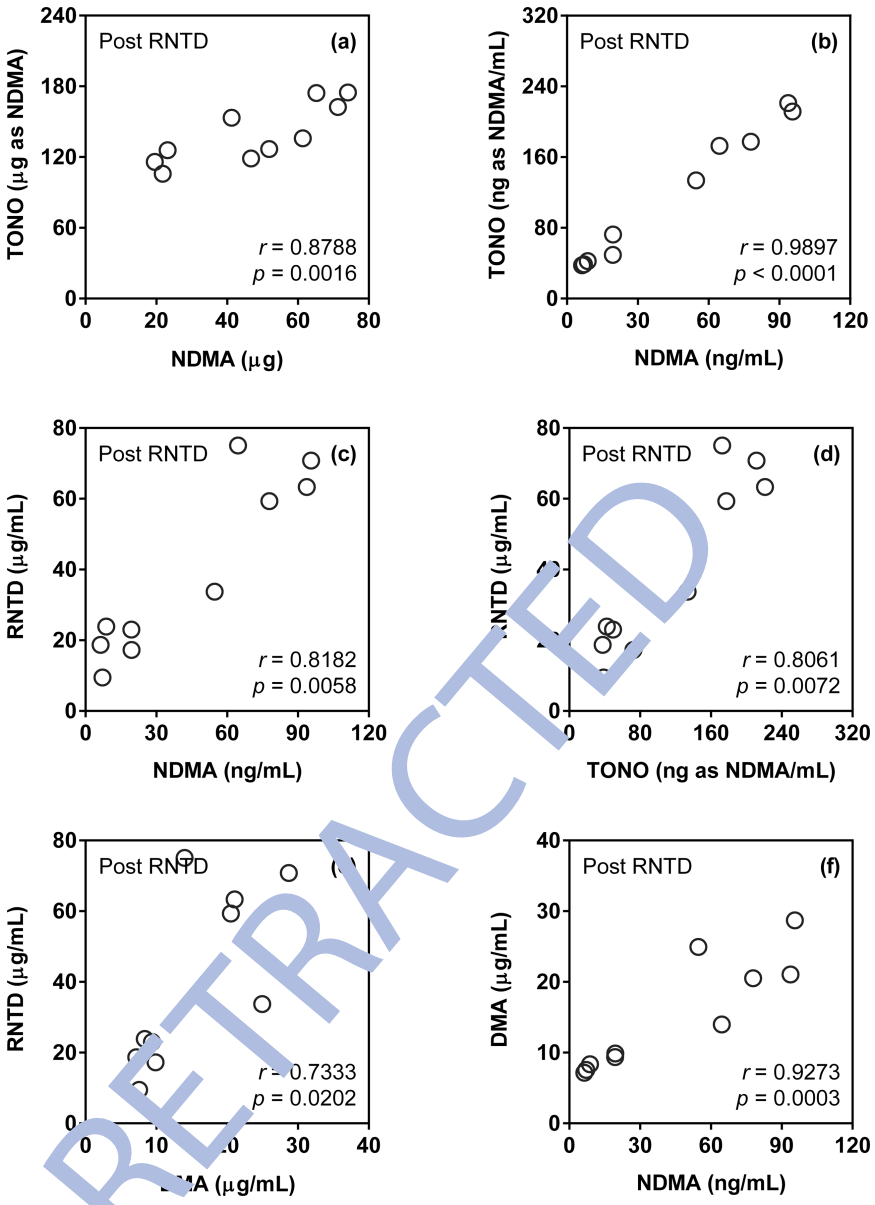


Figure 4. Spearman rank correlation analy between the 24-h excreted masses or mean urinary concentrations of NDMA, TONO, dimethylamine (DMA) and ranitidine (RNTD) after ranitidine intake (i.e. Post RNTD). Correlation analyses for data collected before ranitidine intake (i.e. Pre RNTD) are provided in the Supplementary Figure 4, available at *Carcinogenesis* Online.

(19,20). While urinary *N*-nitrosamine concentrations may more directly reflect systemic exposure, it is important to note that such estimates are conservative. Actual systemic NDMA exposure is likely much higher than that eliminated in urine, as previous studies have indicated a high metabolic conversion rate of NDMA (i.e. >99.9%) and therefore its low renal clearance (i.e. only ~0.05% excreted in urine) (21).

Moreover, the urinary excretion of *N*-nitrosamines after ranitidine intake (139 µg total *N*-nitrosamines expressed as NDMA equivalents and 47.6 µg NDMA) was higher than observed after intake of other pharmaceuticals, and far higher than estimated daily dietary intakes. For example, the maximum excreted mass of *N*-nitrosamines measured in urine after consumption of other pharmaceuticals was 10 µg after consumption of amidopyrine (50). A typical daily dietary intake of NDMA for US adults aged 20–49 years is 60 ng/day (51). A maximum daily drinking water intake of NDMA is 20 ng/day, assuming consumption of 2 L of drinking water containing NDMA at California's 10 ng/l Notification Level for drinking water (52).

The potential cancer risk from ranitidine use should be balanced against its therapeutic benefit. However, due to the widespread use of ranitidine, the increase in urinary NDMA excretion suggests the need for a more comprehensive risk assessment relevant to chronic ranitidine use or the identification of treatment alternatives. Collecting data on NDMA concentrations in other body fluids (e.g. blood) would provide additional constraints for risk estimates. NDMA formation and associated risk might be significantly reduced by administering ranitidine with ascorbic acid that rapidly scavenges nitrite (43,53). The use of

alternative medications, such as proton pump inhibitors (PPIs), would less likely promote *in vivo* nitrosation because of the lack of amines in their structures.

Epidemiological studies evaluating cancer risk, particularly bladder cancer, attributable to the long-term use of ranitidine and structurally similar H_2-receptor antagonists also are needed. These studies should evaluate cancer risk relative to the use of alternative treatments, such as PPIs, to differentiate the risk attributable to the additional loading of *N*-nitrosamine precursors from H_2-receptor antagonists from indirect effects associated with the activity of both H_2-receptor antagonists and PPIs. For example, like H_2-receptor antagonists (54), long-term treatment with omeprazole, the most widely used PPI, promotes endogenous formation of *N*-nitrosamines (55,56), by increasing gastric pH towards levels that either maximize nitrosation reactions (e.g. pH 3.5 (36)) or that promote bacterial colonization and biological catalysis of *N*-nitrosamine formation. A review of epidemiological studies examining long-term use of H_2-receptor antagonists, including ranitidine and cimetidine, found some elevated risk of gastric cancer over the first 5 years of use, but none for use ≥10 years; the apparent elevated risk detected over the early years of use was attributed to lack of detection of pre-existing gastric cancer rather than a true risk attributable to the medications (57). These findings concur with previous studies suggesting only a maximum 6.6-fold increase in NDMA concentrations in gastric fluids resulting from ranitidine consumption (16). The ~400-fold increase in the urinary NDMA concentrations upon ranitidine consumption observed in our study suggests additional formation of NDMA from ranitidine by other pathways within the body (19,20), and the potential for greater cancer risk specific to H_2-receptor antagonists. Interestingly, one epidemiology study of breast cancer noted an increased risk of ductal carcinoma from the use of ranitidine, but not other H_2-receptor antagonists (e.g. cimetidine), despite the fact that cimetidine is far more potent in terms of altering hormone levels by promoting serum prolactin concentrations (58). Regarding bladder cancer, one prospective study observed a potential risk associated with the use of ranitidine or cimetidine (relative risk = 1.58; 95% confidence interval = 0.93–2.69) (54). However, our results suggest a need for additional research on risks associated with long-term usage of ranitidine.

Supplementary material

Supplementary Tables 1–5 and Supplementary Figures 1–4 can be found at http://carcin.oxfordjournals.org/.

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Retraction

RETRACTION

Carcinogenesis has retracted the following article at the request of the authors:

Zeng, T. *et al.* (2016) Oral intake of ranitidine increases urinary excretion of N-nitrosodimethylamine. *Carcinogenesis*, 37, 625–634. https://doi.org/10.1093/carcin/bgw034

This article is being retracted at the request of the authors. Recent research (1) has identified the potential for an analytical artefact associated with the use of gas chromatography that could have contributed to the levels of N-nitrosodimethylamine (NDMA) measured in urine samples containing ranitidine in this study. Given this artefact, the authors have informed the journal that their NDMA measurements are not reliable.

1. United States Food and Drug Administration (FDA). U. S. FDA Updates and Press Announcements on NDMA in Zantac (ranitidine). FDA Provides Update on Testing of Ranitidine for NDMA Impurities. https://www.fda.gov/drugs/drug-safety-and-availability/fda-updates-and-press-announcements-ndma-zantac-ranitidine